RESEARCH AND DEVELOPMENT, NEUCHATEL - QUARTERLY REPORT

DIVISION : RESEARCH

SUBJECT TITLE : EUROP

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KEYWORDS : bacteria, bacillus, biocontrol, rl, sel, preservative, d-alanine, spore, vegetative-cell, germination, park-500, trial

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OBJECTIVE
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To control the germination of bacterial spores during tobacco processing and the bacterial proliferation in the RL process in order to replace traditional preservative systems by a biocontrol agent.

STATUS
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PME trials : simulated SEL

Bacillus pumilus spore germination was measured as a function of incubation temperature. The stability of D-alanine was evaluated over time in simulated SEL extracts. A certain number of substances were analysed before and after germination process in tobacco extracts.
Bacteria isolated in PM-US from laboratory-spoiled Park 500 SEL were identified.

PM-US trials : Park 500 SEL

A series of experiments were conducted in Richmond by M. Hofer in order to evaluate the efficiency of D-alanine in the prevention of Park 500 SEL spoilage.

RESULTS

PME trials

- Germination at different temperatures
  The germination was measured by loss of absorbance at 660 nm after inoculation by B. pumilus spore suspension [1] in SEL extract at 25, 37, 45, 50, 55°C (Fig. 1).
  The faster rate was observed at 37°C with germination completed after 4 hours.
  After 24 hours incubation germination ratio was about 90% at 50°C but was 0% at 55°C.

- D-alanine stability
  Sterile SEL supplemented with 900 mg/l D-alanine was incubated at 37°C. Samples taken over time were inoculated with B. pumilus spores and checked for germination. After 48 hours incubation, germination inhibition was still complete. In trials with physiologically heterogeneous cultures containing not only spores but also some vegetative cells, germination was often detected after 24 hours incubation [2]. This was probably due to disappearance of D-alanine by degradation, transformation or consumption by vegetative cells in growing stage.

- Chemical analysis
  Cut filler and SEL extracts, before and after germination of B. pumilus spores at 37°C, were sent to different laboratories for analysis. The levels of reducing sugars, total alcaloids, NO3, NH3, K, Mg, Ca, Na and amino acids were measured.
  No significant or reproducible changes of any analysed substances were found between the results of analyses before and after germination.

- US strains
  Seven sporulating Gram positive bacteria isolated from laboratory-spoiled Park 500 SEL were tentatively characterized by known morphological comparisons and biochemical tests [3,4].

Isolate code	Strain
8397-70-1	B. circulans
8397-70-2A	--
8397-70-4	B. circulans
8397-70-B	B. circulans
8397-70-C	B. circulans
8397-70-13	B. licheniformis
[redacted]	--

Isolates 8397-70-2A and -8246 could not be identified at this time.
Strain -13 was the only one able to grow in SEL extract at 37°C.

PM-US trials

A double procedure developed by the Project 1730 Research Group was applied to test the efficiency of D-alanine on Park 500 SEL. As efficacy of D-alanine decreases when germinated and vegetative cells are present, it is obvious that D-alanine should be incorporated as soon as possible during feedstock extraction in the RL process. D-alanine will block spore germination activation while, at the same time, hot water kills all germinated and vegetative forms.
As such an evaluation was not possible in Park 500, the procedure was modified by introducing a heat-shock (30-45' at 78°C) of the SEL prior to incubation.

In a first phase, 4 liters of SEL samples from Park 500 line II cleaner feed tank, with and without addition of 1000 mg/l of D-alanine, were incubated at 42°C into bioreactors under 100 RPM stirring. The pH value was recorded over time and samples were taken daily for microbial counting (CFUs = colony forming units) as well as reducing sugar, acetic acid, N-nitrate and soluble ammonia analyses.
D-alanine did not show any effect in the first experiment without heat treatment. Fig. 2 shows that both pH curves are similar, decreasing first, then increasing after 6 hours incubation. These results are confirmed by the bacterial counting (Fig. 3). One morphological type of colony formed appears to increase from the beginning of the incubation (Fig. 4).
As a result of this bacterial development a certain number of chemical changes were analysed in the SEL. Figs. 5 to 8 show the total reducing sugar consumption with production of acetic acid and the utilization of the nitrate through the conversion into soluble ammonia.

In the second experiment with pre-heated SEL, most of the vegetative cells were killed. The inhibition effect on spore germination of D-alanine is illustrated by the pH recording (Fig. 8).
After 24 hours, the pH value of the control version starts changing like the unheated versions of trial 1. In the D-alanine treated version, however, no dramatic change could be recorded after 72 hours.

In a second phase, flasks filled with 100 ml SEL were supplemented with 225, 450, 675 and 900 mg/l D-alanine as well as an untreated control. Two more versions were added to these series : another control and a version with 900 mg/l D-alanine, both pre-heated at 78°C for 30 min. CFUs and pH were measured over time.
Confirming the results of bioreactor assay the unheated versions with different D-alanine concentrations were spoiled like the control after one day.
In the heated versions, the conclusions were also similar. The pH and CFUs changes in the heated versus unheated controls were only delayed for 24 hours. In the 900 mg/l D-alanine version, no changes could be recorded during a 168 hours incubation (Figs. 10 and 11).

The spoilage of the control was attributed to one morphological type of colony (small). The spore germination of this Bacillus type seems to be inhibited by D-alanine (Fig. 12). An attempt to repeat this trial with all D-alanine versions heat-shocked at 78°C for 30 min was unsuccessful. The control as well as all the treated versions were unspoiled after 7 days.

CONCLUSIONS

- A temperature of 55°C, and to a lesser extent 50°C, inhibits germination of B. pumilus spores in SEL.
- The presence of vegetative cells in a culture reduces the efficiency of D-alanine as an inhibitor of spore germination.
- Added to fresh SEL, D-alanine has no effect on bacterial spoilage when incubated in propitious conditions of growing.
- The efficacy of D-alanine was demonstrated in heat-shocked Park 500 SEL.

PLANS

- Study the effects of D-alanine on Bacillus cell physiology during RL feedstock hot extraction.
- Repeat the dose response trial with heat-shocked SEL in order to determine the optimal D-alanine concentration to prevent spoilage.

REFERENCES

[1] Hofer-M., Kälin-P., Quarterly Report Project EUROP, January-March 1988.
[2] Kälin-P., Quarterly Report Project EUROP, July-September 1988.
[3] Gordon-R.E. et al., The Genus Bacillus, CRC Handbook of Microbiol. (1973).
[4] Lemille-F. et al., Essai de classification biochimique de 97 Bacillus, Ann. Institut Pasteur, 116 (1969), 808-19.

EFFECT OF INCUBATION TEMPERATURE ON SPORE GERMINATION IN SEL EXTRACT

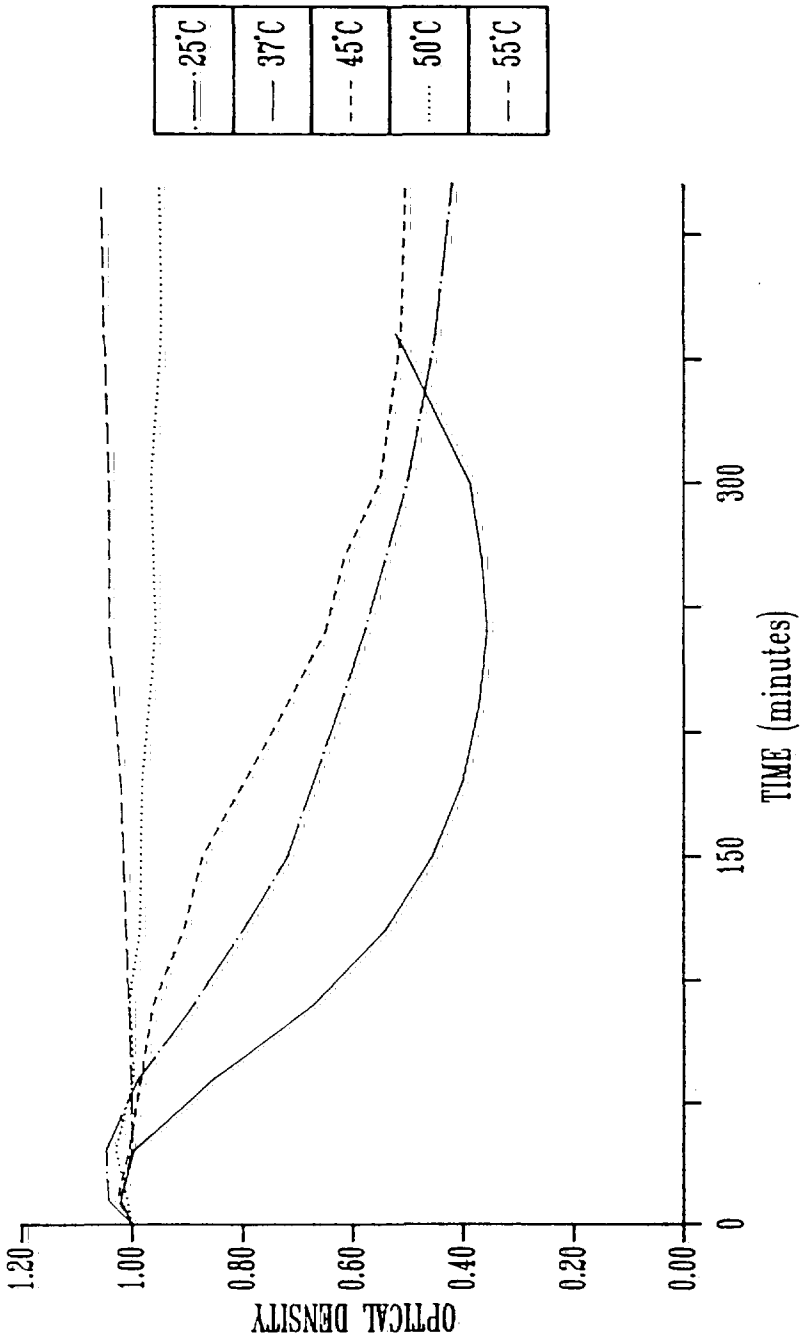

Figure 1

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Figure 2 EFFECT OF D-ALANINE IN PARK 500 SEL
Bioreactor experiment

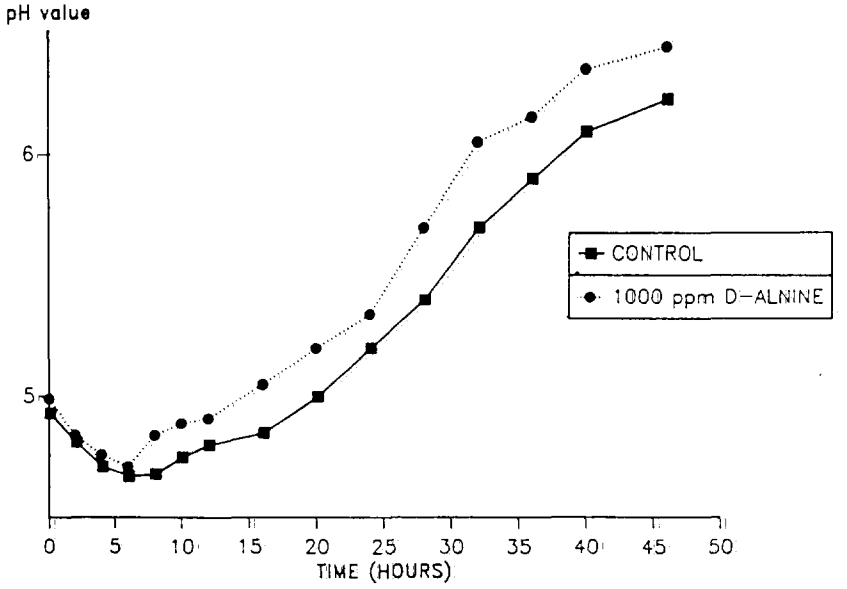

Figure 3 EFFECT OF D-ALANINE IN PARK 500 SEL
Bioreactor experiment

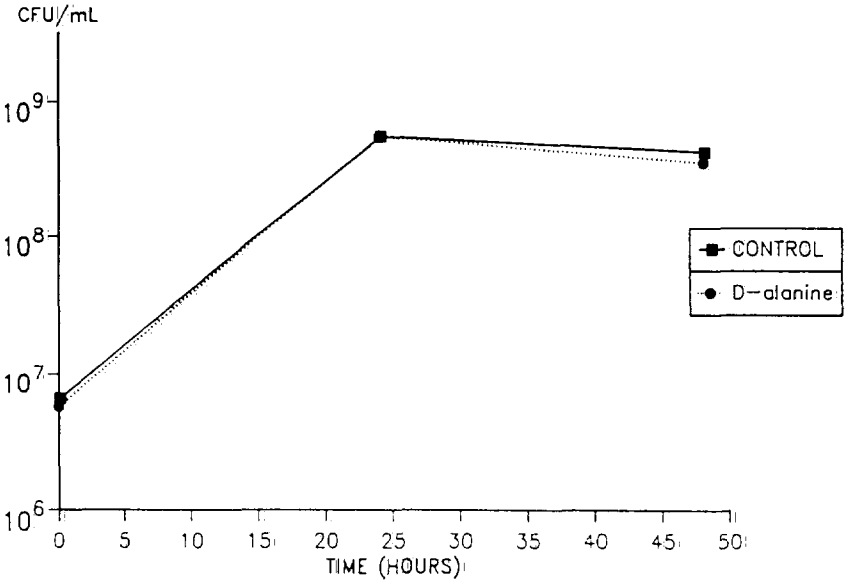

Figure 4

EFFECT OF D-ALANINE ON DIFFERENT CLASSES OF BACTERIA IN SEL
Bioreactor experiment

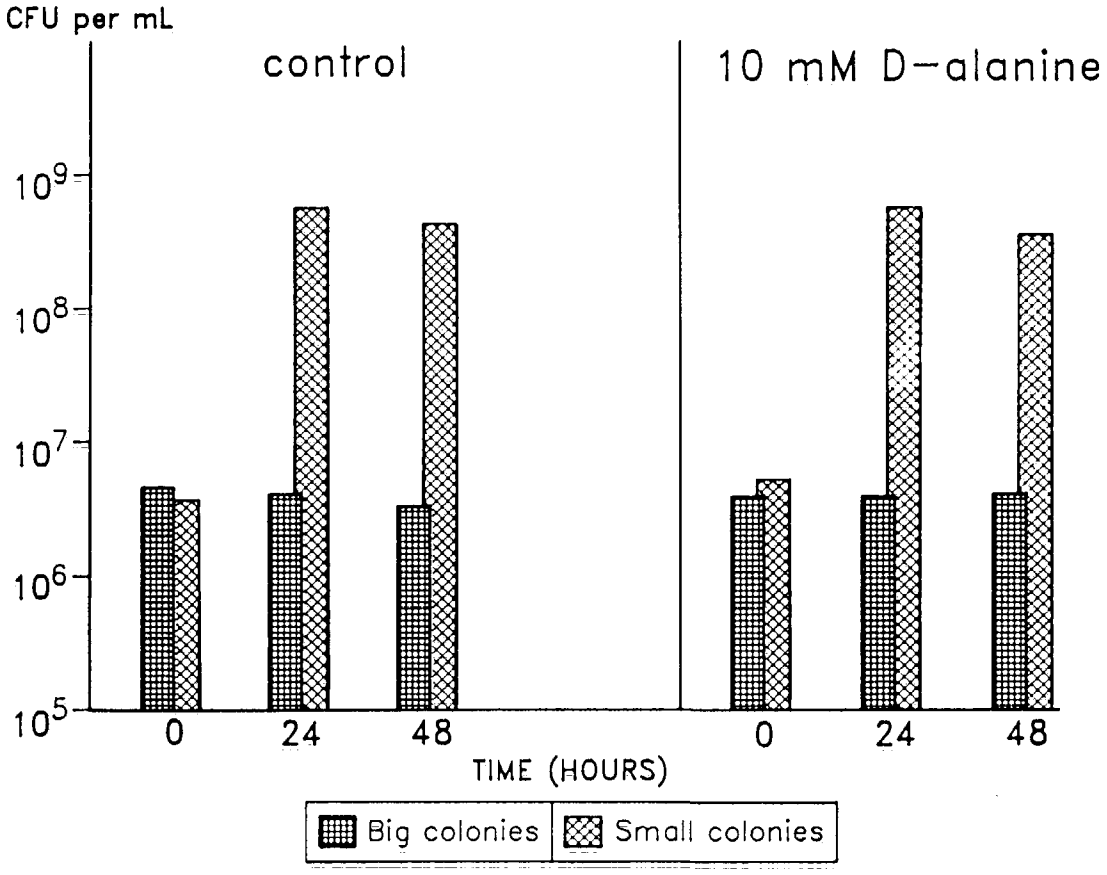

Figure 5 EFFECT OF D-ALANINE IN PARK 500 SEL
Bioreactor experiment

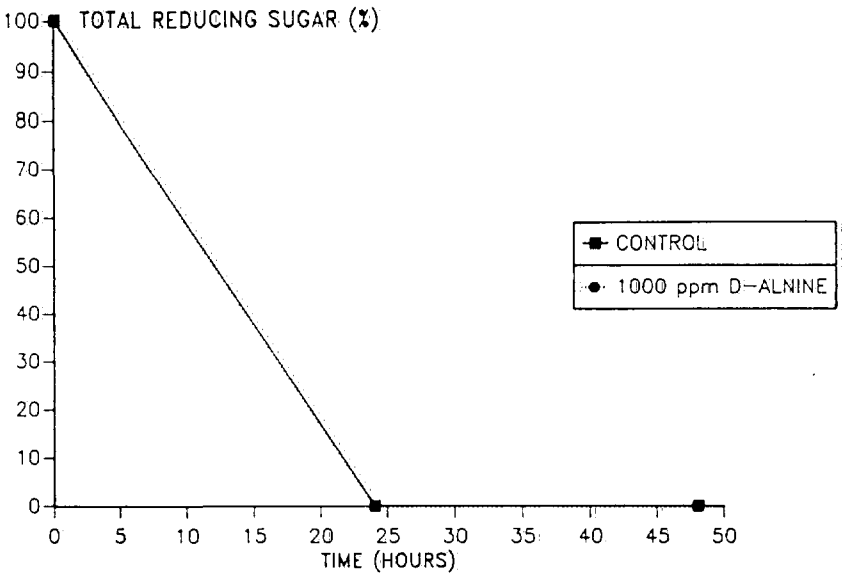

Figure 6 EFFECT OF D-ALANINE IN PARK 500 SEL
Bioreactor experiment

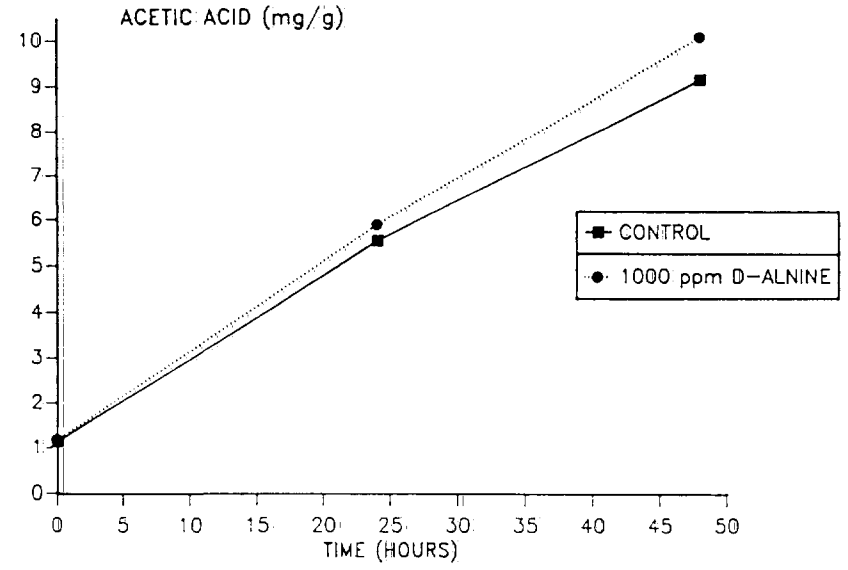

Figure 7 EFFECT OF D-ALANINE IN PARK 500 SEL
Bioreactor experiment

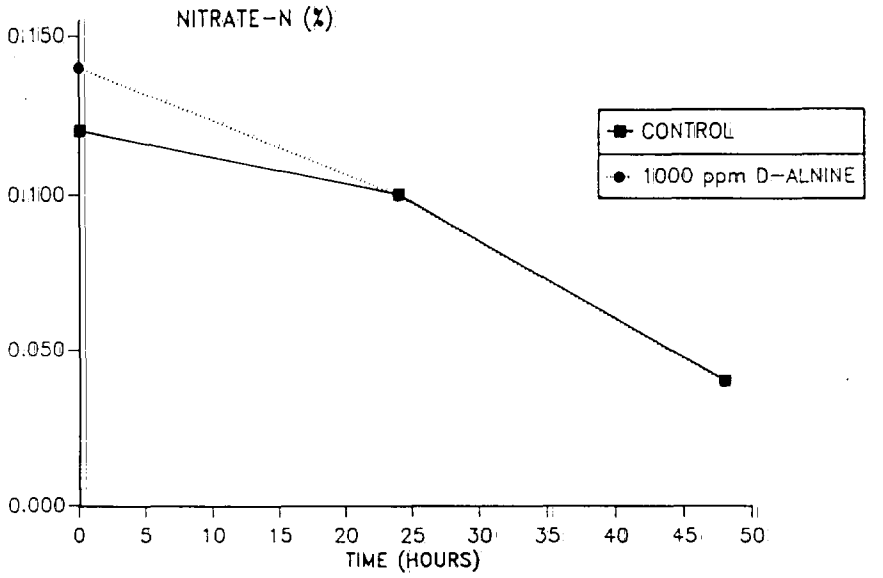

Figure 8 EFFECT OF D-ALANINE IN PARK 500 SEL
Bioreactor experiment

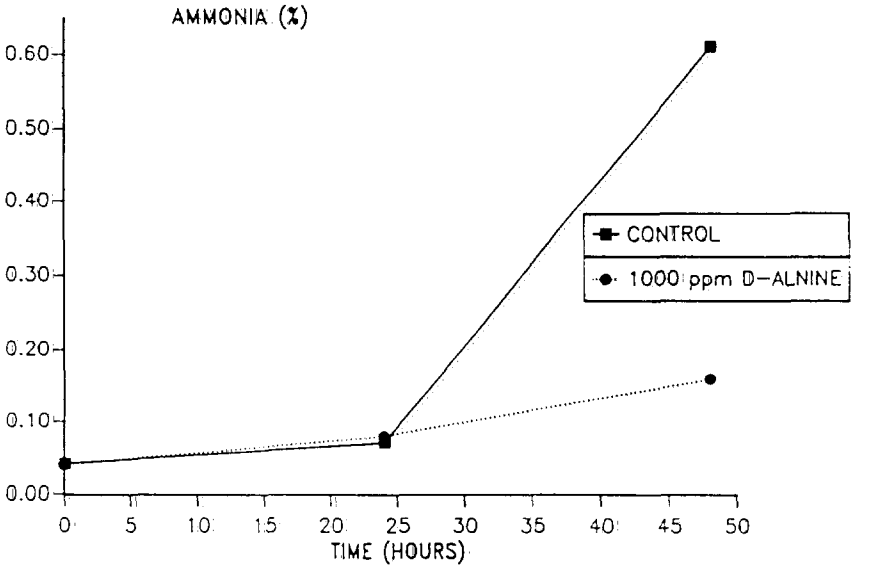

Figure 9

EFFECT OF D-ALANINE IN PARK 500 SEL
Bioreactor experiment

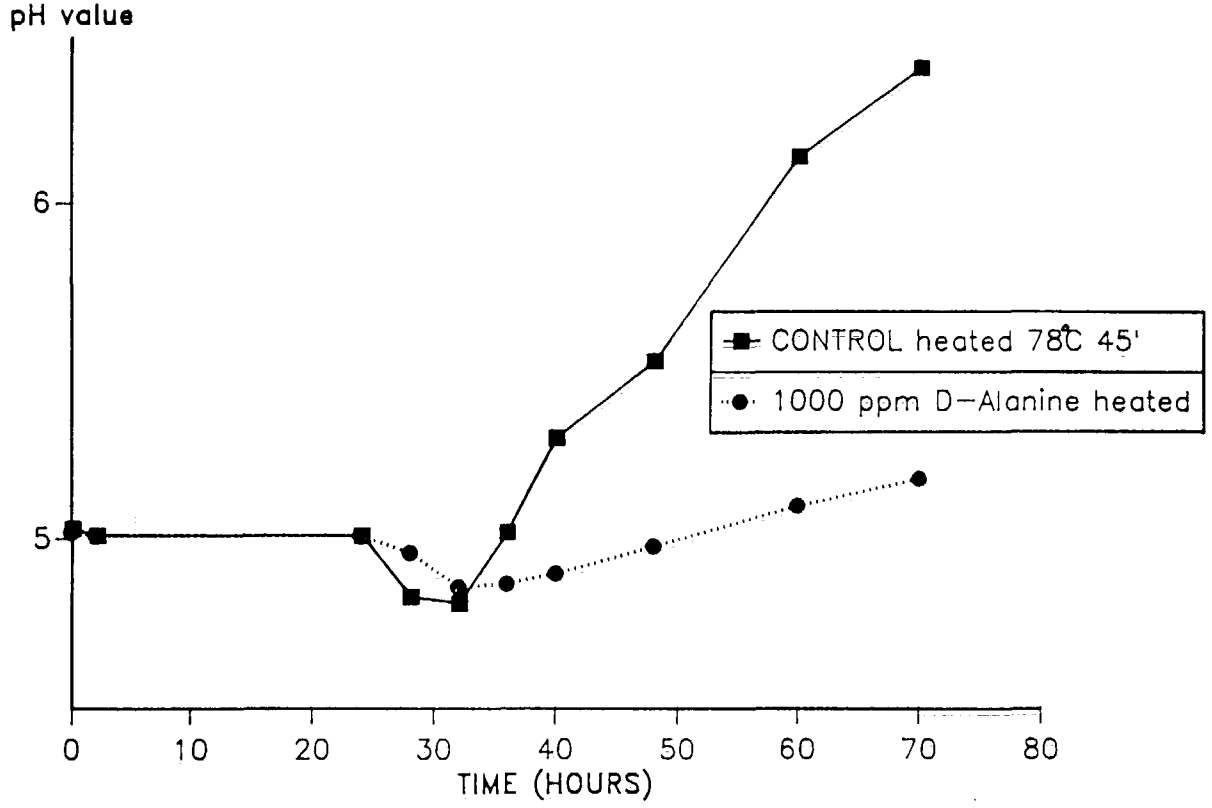

Figure 10 EFFECT OF D-ALANINE IN PARK 500 SEL
flask experiment

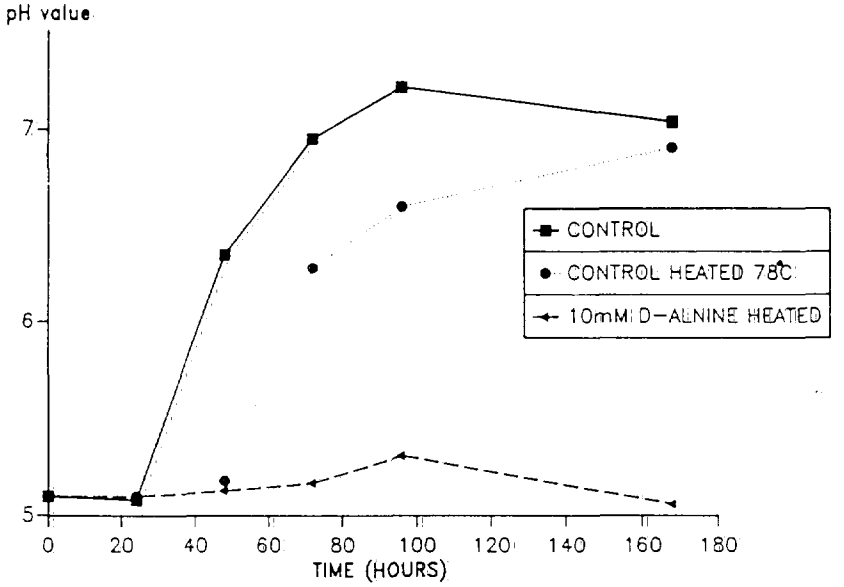

Figure 11 EFFECT OF D-ALANINE IN PARK 500 SEL
flask experiment

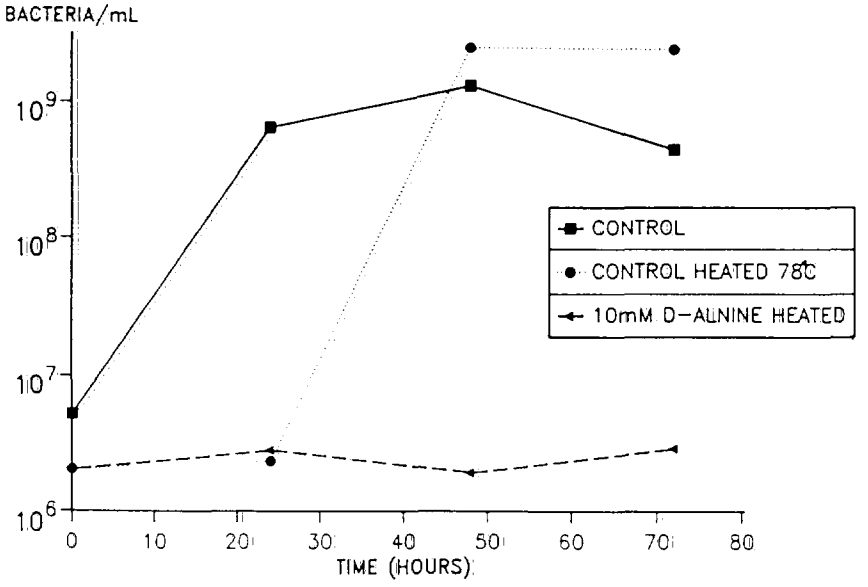

Figure 12

EFFECT OF D-ALANINE ON DIFFERENT CLASSES OF BACTERIA IN SEL
Versions heated at 78°C for 30 minutes